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Prosaposin Facilitates Sciatic Nerve Regeneration In Vivo

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Abstract: Prosaposin, a multifunctional protein, is the precursor of saposins, which activate sphingolipid hydrolases. In addition to acting as a precursor for saposins, prosaposin has been shown to rescue hippocampal CA1 neurons from lethal ischemic damage in vivo and to promote neurite extension of neuroblastoma cells in vitro. Here we show that prosaposin, when added to a collagen-filled nerve guide after sciatic nerve transection in guinea pigs, increased dramatically the number of regenerating nerve fibers within the guide. To identify the target neurons of prosaposin during peripheral nerve regeneration, we determined the degree of atrophy and chromatolysis of neurons in the spinal anterior horn and dorsal root ganglia on the prosaposin-treated and untreated side. The effect of prosaposin on large spinal neurons and small neurons of the dorsal root ganglion was more conspicuous. Subsequent immunohistochemistry demonstrated that the atrophy of cholinergic large neurons in the anterior horn is prevented to significant extent by prosaposin treatment. These findings suggest that prosaposin promotes peripheral nerve regeneration by acting on α-motor neurons in the anterior horn and on small sensory neurons in the dorsal root ganglion. The present study raises the possibility of using prosaposin as a tool for the treatment of peripheral nerve injuries. **Key Words:** Prosaposin—Neurotrophic—Nerve regeneration—Motor neuron—Dorsal root ganglion.
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Saposins are small glycoproteins that activate hydrolysis by specific hydrolases in lysosomes (O'Brien and Kishimoto, 1991; Fürst and Sandhoff, 1992). Analysis of the cDNA for saposins has demonstrated that there is a precursor protein, named prosaposin, that contains four saposin domains (O'Brien et al., 1988; Nakano et al., 1989; Roman and Grabowski, 1989). Besides its role as the precursor of saposins in lysosome, prosaposin may act as a secretory protein in human milk, CSF, and seminal plasma (Hineno et al., 1991; Kondoh et al., 1991; Hiraiwa et al., 1993). Prosaposin, the precursor of saposin, is present at high concentrations in the brain and muscle, whereas processed saposins rather than prosaposin are found predominantly in the spleen, liver, and kidney (O'Brien et al., 1988; Sano et al., 1989). In the brain, prosaposin is localized exclusively to certain neurons and nerve fibers (Kondoh et al., 1993).

Recently, we demonstrated that prosaposin has a potent ability to rescue hippocampal neurons from lethal ischemic damage in vivo (Sano et al., 1994). O'Brien et al. (1994) demonstrated that prosaposin stimulates neuritogenesis and increases choline acetyltransferase activity in neuroblastoma cells. Because the mRNA for prosaposin is abundant in the dorsal root ganglion (DRG) as well as in the brain during embryonic development, prosaposin may play some pathophysiological roles not only in the brain but also in peripheral nerves (Sprecher-Levy et al., 1993).

In the present study, we examined the in vivo effect of prosaposin on peripheral nerve regeneration.

MATERIALS AND METHODS

Animals

Thirty-two male guinea pigs, weighing 200–250 g, were housed at constant temperature (22°C), with a 12-h light/dark cycle, and given food and water ad libitum. The following experiments were conducted in accordance with the *Guide for Animal Experimentation* at Ehime University School of Medicine.

Prosaposin

Prosaposin was purified from human milk by using affinity chromatography with a monospecific antibody as described previously (Kondoh et al., 1993).

Surgical procedures

The animals were anesthetized by intraperitoneal injection of chloral hydrate (350 mg/kg). Under aseptic conditions, both sides of the sciatic nerves were transected at the level of the midthigh and 2-mm nerve segments were removed. The removed nerve segments were fixed immediately with 4% paraformaldehyde and 3% glutaraldehyde in 0.1 *M* phos-

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Abbreviations used: BSA, bovine serum albumin; ChAT, choline acetyltransferase; DRG, dorsal root ganglion; NSS, normal swine serum; PB, phosphate buffer; PBS, phosphate-buffered saline; TX, Triton X-100.

phate buffer (PB) (pH 7.4), postfixed with osmium tetroxide, and embedded in epoxy resin. These segments served as a preoperative control. Sterile silicone chambers (2.0 mm i.d., 3.2 mm o.d., Dow Corning) were sutured to the epineurium of the proximal and distal ends of the sciatic nerve with 8-0 nylon suture under a surgical microscope. The chambers were 10 mm in length, and ~2-mm stumps of the proximal and distal nerve ends were positioned inside the chamber to give a total gap across the chamber of ~6 mm. On one side, the nerve chamber was filled with 24 μl of 0.3% collagen solution (pH 3), 3 μl of 10× minimum essential medium, 1.5 μl of 0.2 *M* NaOH, plus 1.5 μl of prosaposin (final concentration: 2, 10, 20, 40, and 200 ng/ml) dissolved in phosphate-buffered saline (PBS). On the other side, the vehicle was infused. The collagen solution, when infused into the nerve guide, changed to a gel at body temperature. The ends of the chamber were plugged with silicone to prevent leakage of the contents. The skin was sutured with 4-0 nylon suture (see Fig. 1).

Perfusion fixation

Three weeks later, the animals were anesthetized with chloral hydrate (600 mg/kg) and perfused, first with 250 ml of 0.1 *M* PBS, then with 350 ml of ice-cold fixative containing 4% paraformaldehyde and 3% glutaraldehyde in 0.1 *M* PB (pH 7.4).

Microscopic observation

After perfusion, silicone chambers containing the regenerating sciatic nerves, spinal cord from L6–S1 dorsal root, and DRGs at the levels of L-6 and S-1 were dissected out.

Regenerating nerve fibers were cut transversely at the midportion of the chamber and fixed overnight with the same solution as that used for perfusion–fixation. Subsequently, the nerves were postfixed with 2% osmium tetroxide, dehydrated, and embedded in epoxy resin. Semithin cross sections 0.5 μm thick were collected from the distal end of the proximal half of each regenerate and stained with toluidine blue.

Cross sections of the midportion of preoperative control nerves and regenerated nerves were photographed and the contour of the epineurium was traced on tracing paper. The areas of regenerating nerve fibers were then measured by use of a digitizing tablet.

For quantification of the numbers of regenerating nerve fibers, the cross sections were photographed and printed at a final magnification of ×400. The prints were matched together to form a montage of each cross section, and all nerve fibers were counted under a light microscope (at ×1,000). The proportion of regenerated area or nerve fibers to preoperative control area or nerve fibers was calculated.

Spinal cords and DRGs of the animals treated with prosaposin at a concentration of 20 ng/ml were fixed in 3% formaldehyde in 0.1 *M* PB for >24 h and embedded in paraffin. The spinal cords were sectioned at 5 μm and DRGs at 7 μm. The specimens were stained with cresyl violet. The numbers of neurons in the anterior horn and DRG with discernible nucleoli were counted on every 10th section (at ×400). Areas of motor neurons in the anterior horn with discernible nucleoli on layers 7, 8, and 9, in every 10th section, and areas of neurons in DRG with discernible nucleoli, in every 20th section, were measured with the same digitizing tablet as described above.

To evaluate the effect of prosaposin on chromatolysis of the neurons, we graded chromatolysis as follows: grade 3, Nissl bodies are completely degenerative and the cell body expands; grade 2, Nissl bodies in a portion of the cell body are completely degenerative; grade 1, Nissl bodies tend to degrade; grade 0, normal appearance. Three male control guinea pigs, the same age as the experimental animals, were used to compare chromatolysis of the animals that did undergo surgery with that of normal control animals that did not undergo surgery.

Immunohistochemistry

Spinal cords and DRGs from the animals treated with prosaposin at a concentration of 20 ng/ml were fixed with 3% formaldehyde in 0.1 *M* PB for >24 h, embedded in 5% agarose (type 7) in 0.1 *M* PB, and sectioned at 50 μm. The sections were washed three times in 0.2 *M* PB and heat treated at 95°C for 30 min. They were (1) blocked with 5% bovine serum albumin (BSA), 1% normal swine serum (NSS), 0.1% Triton X-100 (TX), and 0.1% NaN_3 in 0.1 *M* PBS overnight at 4°C; (2) incubated with 0.25% carrageenan, 5% BSA, 1% NSS, 0.1% TX, and 0.1% NaN_3 in 0.1 *M* PBS for 1 h at 4°C; (3) incubated with rabbit polyclonal antibody (IgG) against choline acetyltransferase (ChAT) (Chemicon International Inc.), diluted 1:2,000 in blocking solution or with a substance P antiserum (Sakanaka et al., 1982) diluted 1:1,000 in blocking solution, for 48 h at 4°C; (4) incubated with biotinylated anti-rabbit IgG diluted 1:500 in blocking solution, for 24 h at 4°C; (5) incubated with streptavidin peroxidase, diluted 1:500 in 5% BSA, 0.1% TX in 0.1 *M* PBS, for 24 h at 4°C; and (6) subjected to a modified version of the cobalt–glucose oxidase–diaminobenzidine method (Sakanaka et al., 1982).

Ten sections from each spinal cord at the levels of L-6 and S-1 and each seven sections from both sides of DRGs at the same levels were taken for analysis. In individual sections, α-motor neurons and sensory neurons immunoreactive for ChAT and substance P, respectively, were counted. Areas of immunopositive neurons were measured with the digitizing tablet as described above.

Statistics

Areas and fiber counts of the prosaposin-treated regenerative sciatic nerves were compared with those of the untreated nerves after transection, by using a paired *t* test. The effect of prosaposin on the area and chromatolysis of the neurons was evaluated by one-tailed Mann–Whitney *U* test. Spear-

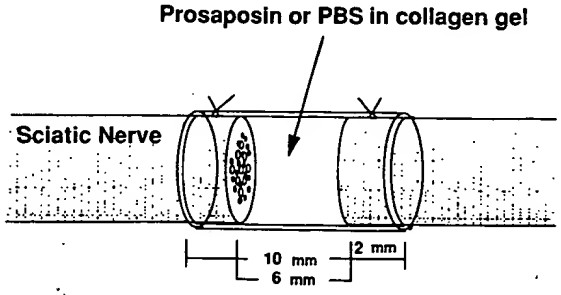

FIG. 1. Schematic of experimental procedures. On one side, the nerve guide is filled with a collagen–prosaposin–PBS mixture in liquid form after sciatic nerve transection. The collagen–PBS mixture is placed on the other side. At body temperature, the mixture gels. The cut ends are plugged with silicone.

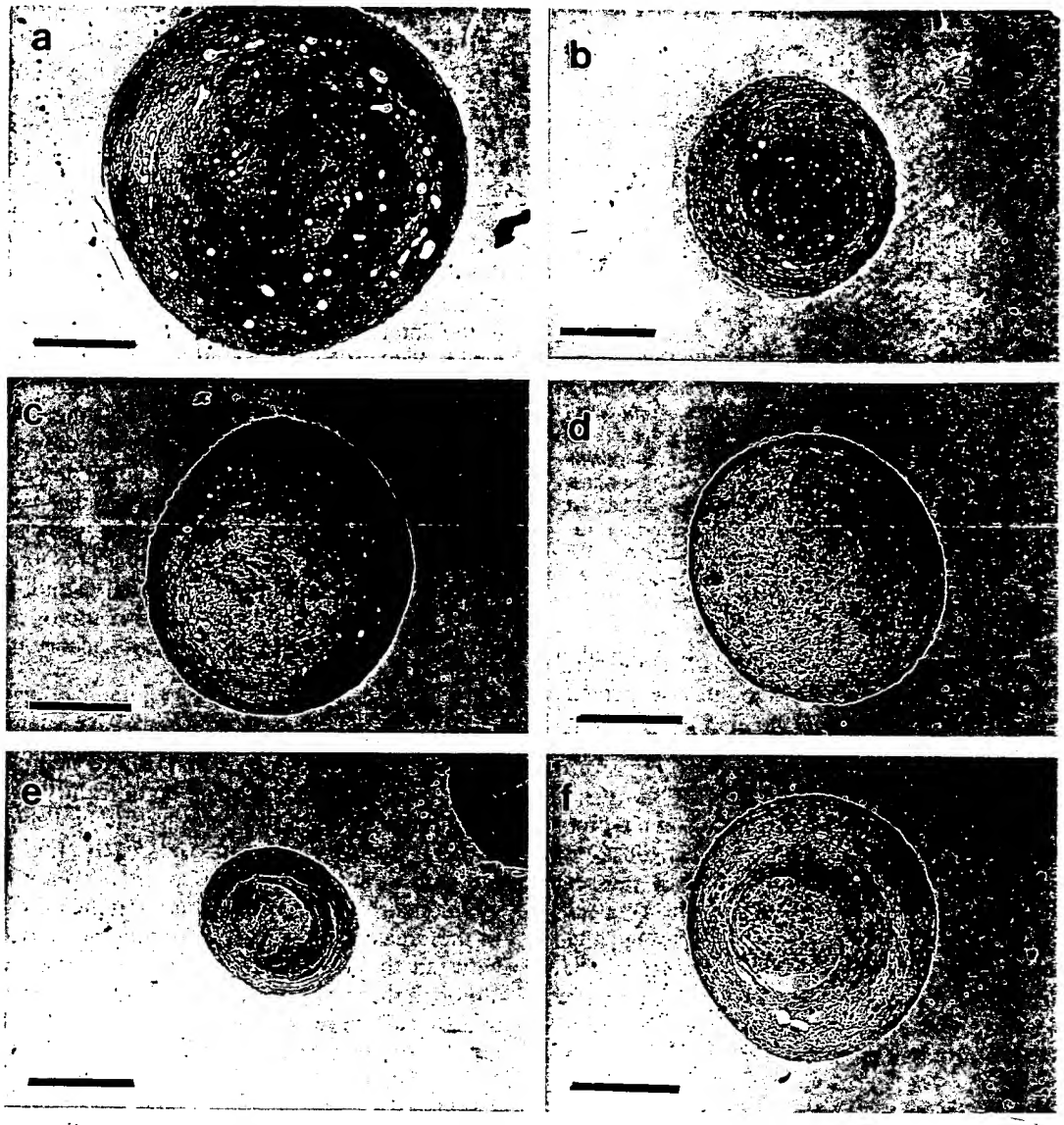

FIG. 2. Cross sections at the midpoint of regenerating nerves, 3 weeks after sciatic nerve transection, and silicone tubulization with or without prosaposin. Photomicrographs of toluidine blue–stained sections. **a:** Prosaposin (20 ng/ml)–treated sciatic nerve. **b:** Vehicle-treated sciatic nerve on the side contralateral to the sciatic nerve of (a). **c:** Prosaposin (40 ng/ml)–treated sciatic nerve. **d:** Vehicle-treated sciatic nerve on the side contralateral to the sciatic nerve of (c). **e:** Prosaposin (200 ng/ml)–treated sciatic nerve. **f:** Vehicle-treated sciatic nerve on the side contralateral to the sciatic nerve of (e). Bar = 500 μm.

man's rank correlation test was used to determine the association between area and chromatolysis of the neurons.

RESULTS

Area of regenerating sciatic nerve

To assess the ability of prosaposin to stimulate peripheral nerve regeneration, we added prosaposin to a collagen-filled nerve guide in vivo after sciatic nerve transection (Fig. 1) and investigated its influence on area and number of regenerating sciatic nerve fibers. As shown in Fig. 2, the area of regenerating nerves in the presence of 20 ng/ml prosaposin was approximately three times larger than that of regenerating nerves not treated with prosaposin ($p < 0.05$, data not shown). However, at a higher concentration (40 ng/ml) of prosaposin, the neurotrophic effect disappeared and 200 ng/ml of prosaposin actually suppressed sciatic nerve regeneration.

Number of axons

Prosaposin caused a dose-dependent increase in the ratio of number of regenerating nerve fibers to number of preoperative nerve fibers (Fig. 3). When the nerve guide was filled with 20 ng/ml prosaposin, there was a statistically significant increase in the ratio (prosa-

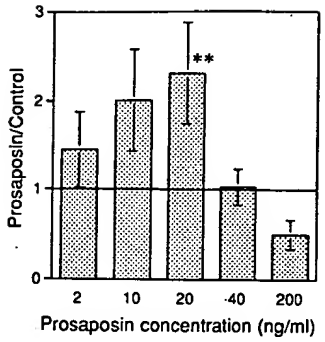

FIG. 3. Effect of prosaposin on the number of regenerating sciatic nerve fibers. The procedure of calculation was as follows: The ratio of the number of regenerated fibers to that of preoperative control fibers was calculated on both sides of the sciatic nerves in individual animals; then the ratio on the prosaposin-treated side was divided by that of the vehicle side. Prosaposin caused a dose-dependent increase in regenerated fibers. An excessive dose (200 ng/ml) of prosaposin suppressed the regeneration of sciatic nerve. Each value represents mean ± SE (n = 5–7), $^{**}p < 0.01$.

posin per vehicle, 2.30 ± 0.57, $p < 0.01$). However, the ratio declined at higher concentrations, as expected from the effect of prosaposin on the area of nerves to which it was applied.

Target neurons

Spinal motor neurons and DRG neurons on the prosaposin-infused side were slightly larger than those on the vehicle side and showed chromatolysis to a lesser degree (Figs. 4 and 5). When the area of neurons in the anterior horn and DRG was morphologically determined in the samples infused with prosaposin at a concentration of 20 ng/ml and in those treated with the vehicle, there was a significant difference between the two sample groups (prosaposin vs. vehicle in the anterior horn, 614 ± 17 vs. 572 ± 18 μm^2, $p < 0.01$; prosaposin vs. vehicle in the DRG, 702 ± 12 vs. 651 ± 11 μm^2, $p < 0.01$). Neuronal chromatolysis was also more severe on the vehicle side than on the prosaposin-treated side in both the anterior horn and DRG (prosaposin vs. vehicle in the anterior horn, 1.33 ± 0.03 vs. 1.75 ± 0.03, $p < 0.01$; prosaposin vs. vehicle in the DRG, 1.62 ± 0.03 vs. 1.94 ± 0.03, $p < 0.01$). However, prosaposin treatment did not affect the number of neurons either in the anterior horn or in the DRG. We then studied what types of neurons in the anterior horn and DRG were affected by prosaposin. A significant correlation between area and chromatolytic grade of the neurons was found for the anterior horn ($r = 0.763$, $p < 0.05$) and there was a significant negative correlation between those for DRG ($r = 0.766$, $p < 0.05$). So, in larger neurons in the anterior horn and smaller neurons in the DRG, chromatolysis was prevented more effectively by prosaposin treatment. These results suggest that prosaposin, when added to the regenerating sciatic nerve end, prevented atrophy and chromatolysis of the α-motor neurons in the spinal cord and those of small sensory neurons in the DRG.

Immunohistochemistry

The area of spinal ChAT-positive neurons on the prosaposin-treated side was significantly larger than that on the vehicle side (prosaposin vs. vehicle, 1,479 ± 29 vs. 1,262 ± 26 μm^2, $p < 0.01$, Fig. 6a). This reinforces the view that prosaposin prevents atrophy of motor neurons. There were no significant differences in the areas of substance P–immunoreactive neurons between the prosaposin-treated and untreated DRGs, although the mean value was higher on the prosaposin-treated side (prosaposin vs. PBS, 897 ± 30 vs. 831 ± 29 μm^2; Fig. 6b).

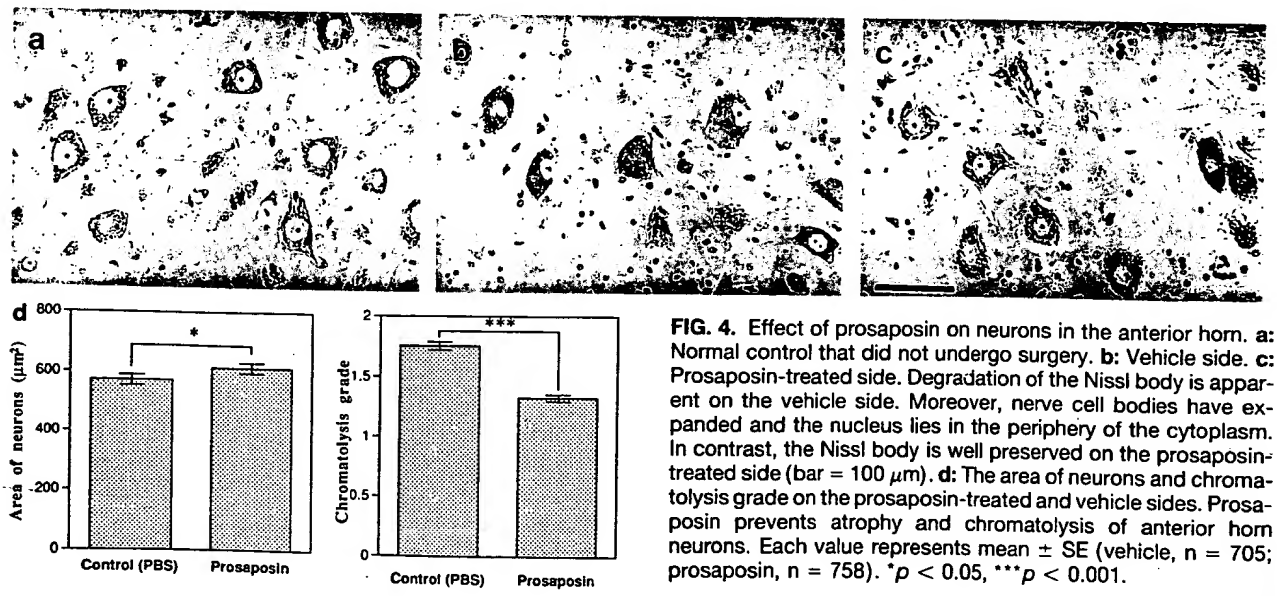

FIG. 4. Effect of prosaposin on neurons in the anterior horn. **a:** Normal control that did not undergo surgery. **b:** Vehicle side. **c:** Prosaposin-treated side. Degradation of the Nissl body is apparent on the vehicle side. Moreover, nerve cell bodies have expanded and the nucleus lies in the periphery of the cytoplasm. In contrast, the Nissl body is well preserved on the prosaposin-treated side (bar = 100 μm). **d:** The area of neurons and chromatolysis grade on the prosaposin-treated and vehicle sides. Prosaposin prevents atrophy and chromatolysis of anterior horn neurons. Each value represents mean ± SE (vehicle, n = 705; prosaposin, n = 758). $^{*}p < 0.05$, $^{***}p < 0.001$.

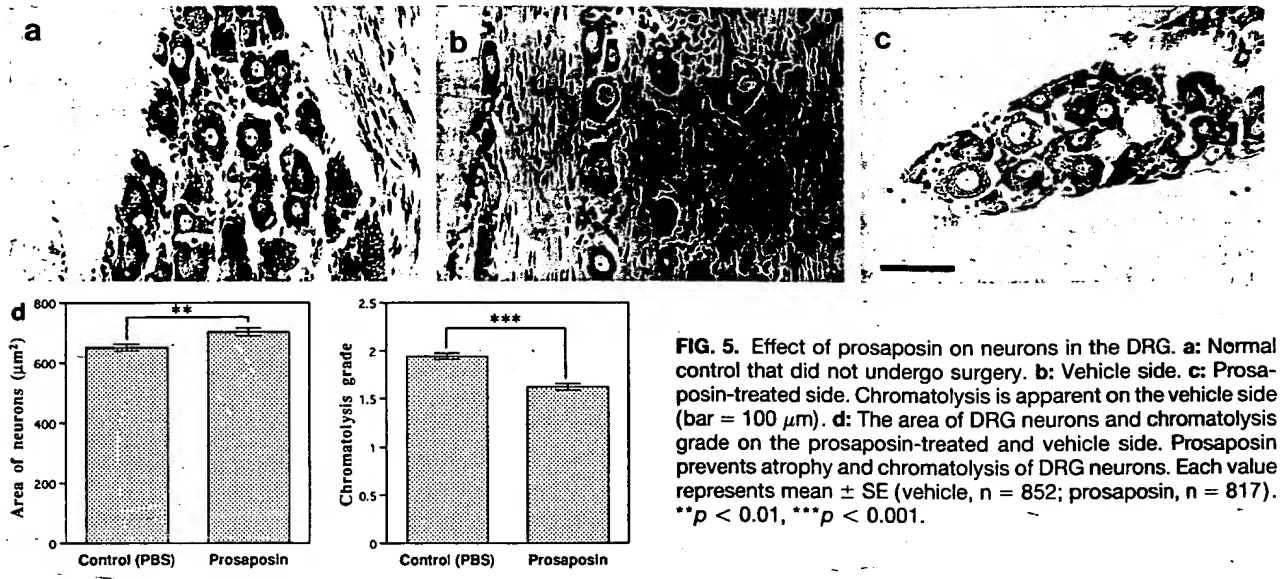

FIG. 5. Effect of prosaposin on neurons in the DRG. **a:** Normal control that did not undergo surgery. **b:** Vehicle side. **c:** Prosaposin-treated side. Chromatolysis is apparent on the vehicle side (bar = 100 μm). **d:** The area of DRG neurons and chromatolysis grade on the prosaposin-treated and vehicle side. Prosaposin prevents atrophy and chromatolysis of DRG neurons. Each value represents mean ± SE (vehicle, n = 852; prosaposin, n = 817). $^{**}p < 0.01$, $^{***}p < 0.001$.

DISCUSSION

Prosaposin is abundant in its unprocessed form in the brain and CSF (Sano et al., 1989; Hineno et al., 1991). Immunohistochemical studies have demonstrated prosaposin-like immunoreactivity specifically in certain neurons of discrete brain regions (Sprecher-Levy et al., 1993). Furthermore, a human baby with a point mutation at the start codon of the prosaposin gene suffered from severe neurological deficits and exhibited prosaposin deficiency (Harzer et al., 1989; Schnabel et al., 1992). These findings suggest pivotal roles of prosaposin in the development of the nervous system. Recently, prosaposin was identified as a neurotrophic factor capable of rescuing hippocampal neurons from lethal ischemic damage in vivo and of inducing differentiation of neuroblastoma cells in vitro (O'Brien et al., 1994; Sano et al., 1994). Because northern and in situ analyses demonstrated an intense expression of prosaposin mRNA not only in the brain and spinal cord but also in the DRG of mouse embryos, the neurotrophic action of prosaposin on peripheral nerves has not been ascertainable.

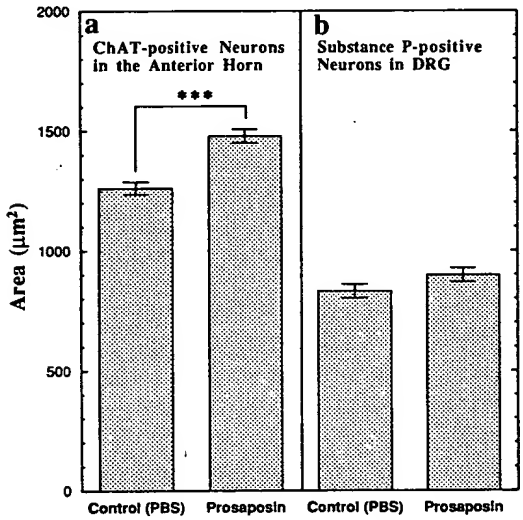

FIG. 6. Effect of prosaposin on the area of ChAT- or substance P–positive neurons. **a:** The area of ChAT-positive spinal motor neurons on the prosaposin-treated and vehicle sides. Prosaposin prevents the atrophy of ChAT-positive neurons. Each value represents mean ± SE (vehicle, n = 244; prosaposin, n = 275). $^{***}p < 0.001$. **b:** The area of substance P–positive DRG neurons on the prosaposin-treated and vehicle sides (vehicle, n = 198; prosaposin, n = 291).

The present study demonstrated that prosaposin can stimulate peripheral nerve regeneration in vivo. The optimal concentration of prosaposin to facilitate nerve regeneration was 20 ng/ml. However, 40 ng/ml prosaposin was ineffective and 200 ng/ml suppressed peripheral nerve regeneration. It is unlikely that the decline in the neurotrophic potency of prosaposin at the higher concentrations is due to an inhibitory compound contaminated in the preparation, as the prosaposin preparation used in this study was affinity purified and checked by amino-terminal sequence analysis in terms of the homogeneity with authentic prosaposin (Kondoh et al., 1993). We showed a relatively narrow concentration dependency of the neurotrophic effects of prosaposin on peripheral nerve regeneration. Similar results have been reported in previous studies dealing with the neurotrophic actions of other trophic factors such as basic fibroblast growth factor, interleukin-1 and 3, and tumor necrosis factor-α (Morrison et al., 1986; Rousselet et al., 1988; Araujo and Cotman, 1991, 1993). The neurotoxic actions of high concentrations of trophic factors including prosaposin may be explained, in part, by the rapid down-regulation of their receptors.

In the present study, experimental nerve guides were filled with collagen gel. After neutralization with phosphate buffer, the collagen solution with or without prosaposin was infused into the nerve guides and the collagen formed a gel at body temperature. Because of the apparent impurity of the collagen preparation, the gel was assumed to contain contaminant proteins in addition to collagen per se. Therefore, we chose the collagen gel without prosaposin as a control to subtract the effects of collagen and other ingredients on peripheral nerve regeneration. The optimal concentration of prosaposin (20 ng/ml) for promoting peripheral nerve regeneration is apparently lower than those of neurotrophic factors examined thus far such as nerve growth factor and acidic fibroblast growth factor (Morrison et al., 1986; Chen et al., 1989; Cordeiro et al., 1989; Rich et al., 1989; Araujo et al., 1993; Derby et al., 1993). We speculate that prosaposin is a potent neurotrophic factor that facilitates nerve regeneration in vivo. In our previous study, prosaposin but not BSA prevented the occurrence of ischemia-induced learning disability and neuronal loss (Sano et al., 1994). These findings may reinforce the specificity of prosaposin action.

To identify neuronal cell types responsive to prosaposin, we studied morphological changes in spinal motor neurons and DRG neurons. Transection of the sciatic nerve caused atrophy and chromatolysis of these neurons, and prosaposin precluded the degenerative processes of spinal and DRG neurons. The prosaposin-responsive neurons in the anterior horn were large ChAT-positive neurons, i.e., α-motor neurons and small DRG neurons with substance P immunoreactivity. These results indicate that prosaposin is a neurotrophic factor with a potent ability to promote peripheral nerve regeneration by acting on α-motor neurons. However, it remains to be determined what types of neurons, other than substance P–containing neurons, are supported by prosaposin.

Although the molecular mechanisms underlying the neurotrophic action of prosaposin are not clear at present, O'Brien et al. (1994) suggest the presence of a putative prosaposin receptor on the surface of neuroblastoma cells. Using ^{125}I-prosaposin, we checked the movement of prosaposin that was infused into a nerve guide; even 7 days later, no movement was observed (data not shown). This indicates prosaposin acts on the cut end of peripheral nerves, without internalization. In conclusion, the present study raises the possibility of prosaposin being used as a tool for the treatment of peripheral nerve damage.

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